Interaction between probiotic lactic acid bacteria and canine enteric pathogens: a risk factor for intestinal Enterococcus faecium colonization?

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Abstract

Selected probiotic lactic acid bacteria (LAB) have been shown to elicit positive health effects particularly in humans. Competitive exclusion of pathogens is one of the most important beneficial health claims of probiotic bacteria. The effect of probiotic LAB on competitive exclusion of pathogens has been demonstrated in humans, chicken and pigs. In this study we evaluated the ability of certain LAB strains (Lactobacillus rhamnosus GG, Bifidobacterium lactis Bb12, Lactobacillus pentosus UK1A, L. pentosus SK2A, Enterococcus faecium M74 and E. faecium SF273) to inhibit the adhesion of selected canine and zoonotic pathogens (Staphylococcus intermedius, Salmonella Typhimurium ATCC 14028, Clostridium perfringens and Campylobacter jejuni) to immobilised mucus isolated from canine jejunal chyme in vitro. Adhesion of C. perfringens was reduced significantly by all tested LAB strains, between 53.7 and 79.1% of the control without LAB, the LAB of canine origin yielding the best reduction. The adhesion of S. Typhimurium and S. intermedius were not significantly altered by any of the LAB included in the study. Both enterococci tested significantly enhanced the adhesion of C. jejuni, to 134.6 and 205.5% of the control without LAB. E. faecium may thus favor the adhesion and colonization of C. jejuni in the dog’s intestine, making it a potential carrier and possibly a source for human infection. Enhanced C. jejuni adhesion is a new potential risk factor of enterococci. Our results further emphasize the importance of safety guidelines to be established for the probiotics intended for animal use.

Keywords: Dog; Probiotic lactic acid bacteria; Competitive exclusion
1. Introduction

Probiotics have been defined as “‘microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host’” (Salminen et al., 1999). Selected probiotic lactic acid bacteria (LAB) have been shown to elicit positive health effects in humans (Salminen et al., 1998). While many different ways of action have been proposed by which probiotics exert their favorable effects, adhesion to the intestinal mucosa is considered one of the main mechanisms. It is regarded to be important for transient colonization (Alander et al., 1999), enhanced healing of the damaged gastric mucosa (Elliott et al., 1998), modulation of the immune system (Schiffrin et al., 1997; O’Halloran et al., 1998) and antagonism against pathogens (Jin et al., 2000). Competitive exclusion of pathogens is one of the most important beneficial health claims of probiotic bacteria (Adlerberth et al., 2000; Rolfe, 2000; Reid and Burton, 2002). The effect of probiotic LAB on competitive exclusion of pathogens has been demonstrated with human mucosal material in vitro (Tuomola et al., 1999), and in vivo in chickens (Hirn et al., 1992; Pascual et al., 1999) and pigs (Genovese et al., 2000).

Probiotics have been suggested to be beneficial in maintaining gastrointestinal health of dogs (Biourge et al., 1998). However, no studies exist concerning the ability of probiotics to hinder the adhesion and colonization of intestinal pathogens in dogs. We have earlier shown that selected probiotics intended for human and animal use, and certain LAB strains isolated from dogs, adhered to immobilised canine intestinal mucus (Rinkinen et al., 2000).

Enteric bacterial pathogens must adhere to and penetrate the protective intestinal mucus layer in order to reach and invade the enterocytes and cause clinical infection (Sylvester et al., 1996). The aim of the current study was therefore to examine whether LAB strains found to adhere to the canine intestinal mucus would inhibit the adhesion of selected canine and zoonotic pathogens to canine small intestinal mucus in vitro.

The in vitro mucus adhesion model was applied in this study. It is particularly suitable for the pathogen exclusion assay, as it is important to prevent the adherence of hostile invaders in the beginning of the colonization process.

For the test, we chose Salmonella enterica serovar Typhimurium ATCC 14028, Clostridium perfringens and Campylobacter jejuni (the latter two of canine origin), which are all known canine intestinal pathogens, Salmonella Typhimurium and C. jejuni being also zoonotic pathogens. Although Staphylococcus intermedius is the most common canine skin pathogen, it was also included as the anal mucosa is a known reservoir for the species (Saijonmaa-Koulumies and Lloyd, 1996). Enterotoxin producing C. perfringens is believed to cause acute and chronic intermittent diarrhoea, which is often treated with antibiotics (Kruth et al., 1989; Twedt, 1992; Songer, 1996).

2. Materials and methods

2.1. Animals

Permanent nipple valves for intestinal access were placed in six healthy beagles (five males, one female) in the mid-jejunum, using a method described previously (Wilsson...
Rahmberg and Jonsson, 1997). Operations were performed 2 years prior to this study, and no alterations in the dog’s health or gastrointestinal function were noticed due to the valve. The dogs had been used only for sampling of jejunal chyme and were not medicated. At the time of the experiment, the dogs were 4 years of age. They were fed canned commercial balanced dog food, the main ingredients of which were cereal, meat, animal derivatives, oils and fats, vegetable protein extract and vegetable derivatives. The composition was as follows: raw protein 9%, raw fat 6%, raw fiber 0.4%, calcium 0.3% and phosphorus 0.25%; moisture 80%. The study was approved by the Helsinki University ethics committee.

2.2. Microorganisms and growth conditions

The strains used and their culture conditions are listed in Table 1. The bacteria were grown from stocks stored at −70 °C in 40% glycerol (1% inoculum). For the competitive exclusion assay (see below) of the canine pathogens, 10 μl/ml tritiated thymidine (methyl-1,2-3H-thymidine, 120 Ci mmol⁻¹) was added to the medium to metabolically radio-label the bacteria. LAB were grown in the absence of tritiated thymidine. For the co-aggregation assay (see below) the canine pathogens were also grown without tritiated thymidine. After growth, the bacteria were harvested by centrifugation (2000 × g), washed twice with phosphate buffered saline (PBS; pH 7.2; 10 mM phosphate) and resuspended in PBS. The absorbance at 600 nm was adjusted to 0.5 ± 0.02 in order to standardise the number of bacteria (10⁷–10⁸ CFU ml⁻¹) before use in the competitive exclusion assay (see below).

S. intermedius was kindly provided by National Veterinary and Food Research Institute, Helsinki, Finland, and the C. jejuni was a generous gift from Professor M.L. Hänninen, Faculty of Veterinary Medicine, Department of Food and Environmental Hygiene, University of Helsinki, Finland.

Table 1
Bacterial strains used and their culture conditions

<table>
<thead>
<tr>
<th>Strain</th>
<th>Origin/product</th>
<th>Growth medium</th>
<th>Atmosphere</th>
<th>Incubation time</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. rhamnosus GG</td>
<td>Gefilus® (Valio Ltd.)</td>
<td>MRS</td>
<td>Anaerobic</td>
<td>O/N</td>
</tr>
<tr>
<td>B. lactis Bb12</td>
<td>(Chr. Hansen A/S)</td>
<td>MRS</td>
<td>Anaerobic</td>
<td>O/N</td>
</tr>
<tr>
<td>L. pentosus UK1A</td>
<td>Dog faeces</td>
<td>MRS</td>
<td>Anaerobic</td>
<td>O/N</td>
</tr>
<tr>
<td>L. pentosus SK2A</td>
<td>Dog jejunal chyme</td>
<td>MRS</td>
<td>Anaerobic</td>
<td>O/N</td>
</tr>
<tr>
<td>E. faecium M74</td>
<td>Lactiferm® (Medipharm AB)</td>
<td>MRS</td>
<td>Anaerobic</td>
<td>O/N</td>
</tr>
<tr>
<td>E. faecium SF273</td>
<td>Biobak® (Biofarm OY)</td>
<td>MRS</td>
<td>Anaerobic</td>
<td>O/N</td>
</tr>
<tr>
<td>C. perfringens PP400</td>
<td>Dog</td>
<td>BHI</td>
<td>Anaerobic</td>
<td>1 day</td>
</tr>
<tr>
<td>S. Typhimurium</td>
<td>ATCC 14028</td>
<td>BHI</td>
<td>Anaerobic</td>
<td>O/N</td>
</tr>
<tr>
<td>C. jejuni 517C/R</td>
<td>Dog</td>
<td>BHI + horse serum</td>
<td>Micro-aerobic</td>
<td>3 days</td>
</tr>
<tr>
<td>S. intermedius EELA 2997/99</td>
<td>Dog</td>
<td>BHI</td>
<td>Anaerobic</td>
<td>O/N</td>
</tr>
</tbody>
</table>

*MRS: de Man, Rogosa, Sharpe broth; O/N: over-night; BHI: brain heart infusion.*
2.3. Mucus preparation

A sample of approximately 8 ml of jejunal chyme was obtained via the valve 2 h postprandially and frozen immediately at −70 °C. Mucus was prepared from the jejunal chyme essentially as described earlier (Kirjavainen et al., 1998; Ouwehand et al., 2001). In brief, jejunal chyme was centrifuged at 12,000 × g to remove particulate matter. Mucus was precipitated from the clear supernatants by dual ethanol precipitation and freeze-dried. Equal amounts of mucus from each dog were pooled and a stock suspension of 5 mg ml⁻¹ in HEPES (N-2-hydroxy-ethylpiperazine-N’-2-ethanesulfonic acid)-Hanks buffer (HH; 10 mM HEPES; pH 7.4) was prepared and stored at −20 °C until use.

2.4. Competitive exclusion assay

Competitive exclusion of the canine pathogens by LAB was determined as described previously (Ouwehand et al., 2001). Briefly, mucus stocks were thawed and centrifuged to remove any precipitate formed during storage and diluted in HH to a concentration of 0.5 mg ml⁻¹. The mucus was passively immobilised on polystyrene microtitre plate wells (Nunc Maxisorp, Roskilde, Denmark) by over-night incubation at 4 °C. Excess mucus was removed by washing twice with HH. LAB without radio-label were allowed to adhere to the immobilised mucus, wells with PBS only served as control. Non-bound LAB were removed by washing twice with HH. Radio-labelled pathogenic bacteria were then added to the wells and incubated for 1 h at 37 °C. Non-bound pathogens were removed by washing twice, and bound bacteria were released and lysed by incubation with 1% SDS–0.1 M NaOH for 1 h at 60 °C. Radioactivity of the lysed suspension was measured by liquid scintillation. The adhesion ratio (%) was calculated by comparing the radioactivities of the bacteria added and bound bacteria. Competitive exclusion was calculated as the percentage of pathogens bound after the initial adhesion of the LAB relative to pathogens bound in the absence of pre-adhered LAB (control).

2.5. Co-aggregation

Because some LAB strains were observed to increase the adhesion of the canine pathogens, possible co-aggregation of the LAB and the canine pathogens was investigated. The co-aggregation test was performed as described earlier (Handley et al., 1987). Bacterial suspensions were prepared as described above. The absorbance at 600 nm was adjusted to 0.5. Canine pathogens were mixed with an equal volume of a LAB strain and incubated for 4 h at 37 °C. Absorbance at 600 nm was determined for the mixture and for the bacterial suspensions alone. Co-aggregation (%) was calculated according to the following equation:

\[
\text{OD}_{600}(\text{pathogen alone} + \text{LAB alone})/2 - \text{OD}_{600}(\text{pathogen + LAB combination}) \times 100%
\]

\[
\text{OD}_{660}(\text{pathogen alone} + \text{LAB alone})/2
\]

where ‘pathogen alone’ and ‘LAB alone’ represent the OD₆₀₀ of the separate bacterial suspensions after 4 h, and ‘pathogen + LAB combination’ represents the OD₆₀₀ of the mixed bacterial suspension after 4 h.
2.6. Statistical analysis

Results from the adhesion experiments are expressed as the average of at least three independent experiments. Each experiment was performed with four parallels to adjust for intra-experimental errors. A non-parametric Wilcoxon signed-rank test was used to evaluate the statistical differences ($P < 0.05$) of competitive exclusion and co-aggregation of each strain in comparison with the control. All statistical analyses were performed with StatView (Abacus, Berkeley, USA).

3. Results

All the tested pathogens showed poor to moderate (1.2–5.1%) adhesion to the canine immobilised intestinal mucus. The average adhesions were as follows: *S. intermedius* 4.5% (2.7–5.6%); *S. Typhimurium* 1.2% (1.1–1.4%); *C. jejuni* 5.1% (3.8–6.0%) and *C. perfringens* 3.0 (1.9–5.5%).

Adhesion of *C. perfringens* was reduced significantly by all tested LAB strains, between 53.7 and 79.1% of the control without LAB (Table 2). When compared with other LAB, the reduction was significantly lower with the strain UK1A, but not with SK2A. Both enterococci tested significantly enhanced the adhesion of *C. jejuni*, to 134.6 and

<table>
<thead>
<tr>
<th></th>
<th>C. perfringens</th>
<th>S. intermedius</th>
<th>S. Typhimurium</th>
<th>C. jejuni</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. rhamnosus</em> GG</td>
<td>79.1 ± 6.5*</td>
<td>112.3 ± 31.2</td>
<td>131.7 ± 36.5</td>
<td>101.9 ± 34.1</td>
</tr>
<tr>
<td>B. lactis Bb12</td>
<td>75.1 ± 7.2*</td>
<td>90.2 ± 22.5</td>
<td>97.7 ± 24.7</td>
<td>94.4 ± 22.8</td>
</tr>
<tr>
<td><em>L. pentosus</em> UK1A</td>
<td>57.0 ± 6.3*</td>
<td>101.8 ± 21.4</td>
<td>103.4 ± 26.9</td>
<td>109.8 ± 22.2</td>
</tr>
<tr>
<td><em>L. pentosus</em> SK2A</td>
<td>53.7 ± 17.2*</td>
<td>87.5 ± 24.6</td>
<td>99.9 ± 27.9</td>
<td>127.0 ± 34.3</td>
</tr>
<tr>
<td><em>E. faecium</em> M74</td>
<td>69.8 ± 9.6*</td>
<td>91.8 ± 23.5</td>
<td>97.1 ± 22.3</td>
<td>134.6 ± 17.4**</td>
</tr>
<tr>
<td><em>E. faecium</em> SF273</td>
<td>78.1 ± 3.0*</td>
<td>82.4 ± 21.2</td>
<td>107.7 ± 29.2</td>
<td>205.5 ± 75.0**</td>
</tr>
</tbody>
</table>

* Significant reduction of pathogen adhesion $P < 0.05$.
** Significant increase in pathogen adhesion $P < 0.05$.

Table 3

Co-aggregation between probiotic bacteria and canine pathogens (average change (%) ± standard deviation)

<table>
<thead>
<tr>
<th></th>
<th>C. perfringens</th>
<th>S. intermedius</th>
<th>S. Typhimurium</th>
<th>C. jejuni</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. rhamnosus</em> GG</td>
<td>−0.3 ± 3.2</td>
<td>−0.1 ± 1.7</td>
<td>−0.1 ± 1.4</td>
<td>6.6 ± 4.5*</td>
</tr>
<tr>
<td>B. lactis Bb12</td>
<td>0.0 ± 2.0</td>
<td>1.1 ± 1.8</td>
<td>−0.1 ± 1.9</td>
<td>4.4 ± 4.1*</td>
</tr>
<tr>
<td><em>L. pentosus</em> UK1A</td>
<td>−0.1 ± 2.1</td>
<td>1.1 ± 0.4*</td>
<td>−0.1 ± 2.1</td>
<td>7.0 ± 4.0*</td>
</tr>
<tr>
<td><em>L. pentosus</em> SK2A</td>
<td>0.1 ± 0.8</td>
<td>1.6 ± 2.5</td>
<td>−0.1 ± 0.9</td>
<td>3.2 ± 5.6</td>
</tr>
<tr>
<td><em>E. faecium</em> M74</td>
<td>−4.5 ± 3.2</td>
<td>2.1 ± 1.4</td>
<td>0.4 ± 1.2</td>
<td>1.9 ± 2.9</td>
</tr>
<tr>
<td><em>E. faecium</em> SF273</td>
<td>−1.9 ± 1.5</td>
<td>−1.6 ± 2.1</td>
<td>−0.2 ± 1.1</td>
<td>0.8 ± 2.8</td>
</tr>
</tbody>
</table>

* Significant co-aggregation $P < 0.05$. 
205.5% of the control without LAB, $P < 0.05$ (Table 2). The adhesion of the other tested pathogens was not significantly affected by the LAB.

Significant co-aggregation ($P < 0.05$) was observed for Lactobacillus rhamnosus GG, Bifidobacterium lactis Bb12 and Lactobacillus pentosus UK1A with C. jejuni (Table 3). L. pentosus UK1A also exhibited co-aggregation with S. intermedius (Table 3). No other significant co-aggregation was observed. Co-aggregation was not related to the enhanced C. jejuni adhesion caused by Enterococcus faecium strains.

4. Discussion

We investigated the ability of certain LAB to interfere with the adhesion of selected canine and zoonotic pathogens. L. rhamnosus GG (human isolate), L. pentosus UK1A (canine faecal isolate), L. pentosus SK2A (canine jejunal isolate), B. lactis Bb12 and two strains of E. faecium (a human and a porcine strain) previously reported to adhere to canine intestinal mucus (Rinkinen et al., 2000) were included in the study together with enteric pathogens C. perfringens, C. jejuni (both canine isolates) and S. Typhimurium ATCC 14028. Canine skin pathogen S. intermedius was also included since it has anal mucosa as an important carriage site (Saijonmaa-Koulumies and Lloyd, 1996).

The results show that all the pathogens tested adhered to the immobilised intestinal mucus in vitro. This suggests that they all have the ability to bind to intestinal mucus, which then assists the pathogens in invading the mucosa.

The in vitro mucus adhesion of C. perfringens was significantly reduced by all tested LAB, which can be expected to lead to lower level of colonization of C. perfringens. Whether this is true in vivo needs to be investigated. If in vivo studies further support the in vitro results, it could imply a potential probiotic use of these strains in diminishing the number of C. perfringens organisms in canine intestinal tract. Reduced C. perfringens adhesion does not appear to be related to the adhesive abilities of the tested LAB. We have earlier shown that the strains from canine origin included in this study (SK2A and UK1A) exhibit a relatively low level of adhesion to intestinal mucus as compared with L. rhamnosus GG and B. lactis Bb12 (Rinkinen et al., 2000). The mechanism for this reduction in pathogen adhesion remains obscure.

The adhesion of S. Typhimurium was not significantly affected by any of the tested LAB, in contrast to the findings of Tuomola et al. (1999). In their study, two strongly adhesive strains of lactobacilli increased in vitro adhesion of S. Typhimurium to human intestinal mucus significantly. L. rhamnosus GG was observed in an earlier study to have a very strong adhesion also on canine intestinal glycoproteins (Rinkinen et al., 2000). This probably indicates that powerful adhesion itself does not enhance the binding of S. Typhimurium, rather the increased binding to mucus is due to an unelucidated mechanism, which could be species specific. The adhesion of S. intermedius was not altered by any of the LAB included in this study. Alarmingly, both Enterococcus strains were observed to significantly enhance the adhesion of C. jejuni. In addition to the food and water borne infections, pets (particularly young animals with diarrhoea) are considered to be a probable source of human C. jejuni infection (Ketley, 1997). Campylobacter species have a typical corkscrew motility that enables them to penetrate through the mucus after they attach
themselves to the mucosal surface (Szymanski et al., 1995; Sylvester et al., 1996). Adhesion to mucus receptors has been postulated to enhance this penetration by allowing the organism to attach on the top of the mucosal layer before traveling through the mucus to underlying intestinal epithelial cells (Sylvester et al., 1996). *C. jejuni* lipopolysaccharide has been documented to be an adhesion enabling the organism to adhere to mucus receptors, but binding to mucus varies among different strains (McSweegan and Walker, 1986). Mucin fucose residues are considered chemotactic for *C. jejuni* (Hugdahl et al., 1988). In our study, the lack of adhesion enhancement with other pathogens could be the result of a different type of mucin adherence and could also be strain-dependent. The mechanism by which the enterococci enhance this first line adhesion of *C. jejuni* requires further investigation.

Acute intestinal disorders of dogs are often treated with probiotics and many of the commercial products contain *Enterococcus* species. The current study indicates that probiotic *E. faecium* may favor the adhesion and colonization of *C. jejuni* in the dog’s intestine. This may make the dog a potential carrier of *C. jejuni* and a possible source for human campylobacteriosis. Enhanced colonization by this potential pathogen could thus be an additional risk factor for enterococci in feed or probiotic use. However, enterococci are widely distributed in nature and are also used in food technology, thus, not all the strains can be considered as a health risk. No probiotic enterococcal infections have been reported in the veterinary medicine, so the risk, according to the present knowledge, appears to be limited. However, the research in probiotic therapy in companion animals has been scarce and it may be speculated that possible enterococcal infections from probiotics may have been overlooked. Furthermore, probiotics are often used on debilitated animals and together with antibiotics. Thus, the potential risk for promoting the growth of a zoonotic pathogen should be weighed with the positive health effects exerted by the probiotic.

Co-aggregation may be one mode of communication between bacteria mediated by adhesions on one bacterial genus or species and equivalent receptors on another. Bacterial co-aggregation is a well-known phenomenon in the oral cavity, where it is considered important for oral biofilm formation. This interaction can lead to rich growth where the bacteria involved would not grow independently (Egland et al., 2001). Some LAB co-aggregated with *Escherichia coli* in the urogenital tract (Reid et al., 1988), and intestinal lactobacilli have also been shown to co-aggregate with enteropathogens; porcine lactobacilli co-aggregated with *E. coli* K88 (Spencer and Chesson, 1994). Co-aggregation is thought to be beneficial if LAB produce antimicrobials since the ability of inhibitor-producing LAB to interact with a pathogen in close vicinity may be an important defense mechanism of normal microflora (Reid et al., 1988). We found that co-aggregation was not likely to be the reason for enhanced binding with enterococci. The canine faecal strain *L. pentosus* UK1A co-aggregated with *S. intermedius*, which is known to have the anal mucosa as a reservoir (Saijonmaa-Koulumies and Lloyd, 1996). This interaction could be of ecological importance for the colonization of a skin pathogen, *S. intermedius* on anal mucosa. In the present study the co-aggregation was in general small, although statistically significant, thus, its biological significance is uncertain.

In conclusion, canine mucus provides a new model for preliminary evaluation of competitive exclusion of canine intestinal pathogens in vitro. The results indicate that the LAB tested may be beneficial in reducing the number of *C. perfringens* organisms in
canine intestine. These LAB should be evaluated further for the treatment and prevention of disease in vivo. However, the observed increase in in vitro mucus adhesion of *C. jejuni* caused by the tested enterococci may be cause for concern. The role of potentially pathogenic *E. faecium* in etiology of disease has been discussed in human medicine, mainly for its ability to rapidly develop and transfer antibiotic resistance (Jett et al., 1994; Donohue et al., 1998; Franz et al., 1999) and cause nosocomial infections (Edmond et al., 1999). Enhanced *C. jejuni* adhesion is a new potential risk factor of enterococci and requires further investigation. Finally, our results emphasize the importance of establishing safety guidelines for probiotics intended for animal use.

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**References**


